HNB-LAMP for SARS-CoV-2 detection using QuickExtract crude lysate (v2)

Introduction

This protocol describes the detection of SARS-CoV-2 using RT-LAMP (Reverse Transcription Loop-mediated Amplification) from crude patient samples inactivated in QuickExtract DNA solution. The protocol was established at the Vienna Bio Center as part of the Vienna Covid-19 Detection Initiative (VCDI). Detailed findings covering the method establishment and experimental validation can be found in our pre-print '**A rapid, highly sensitive and open-access SARS-CoV-2 detection assay for laboratory and home testing**' (https://www.biorxiv.org/content/10.1101/2020.06.23.166397v2)

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Several key considerations before performing this protocol:
A) SARS-CoV-2 is a highly contagious virus and requires personal protection with appropriate safety standards. Users must follow Biosafety level guidelines outline by the CDC https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html

B) Seperate working areas for sample preperation, reaction set-up and analysis are highly recommended. Cleaning work surfaces and pipettes with 5-10% Bleach solution (Caution)

C) Do not open RT-LAMP reactions in order to prevent carry-over cross contamination leading to false positives. The non-colorimetric master mix from NEB (E1700L) does not contain dUTP/thermolabile UDG to prevent cross-contamination; we recommend to add these two components to the reaction mix to lower the risk of cross-reaction contamination.

D) 96-well plates must be properly sealed. This is absolutely crucial to prevent cross-well contamination. Proper plate seals (e.g. tight sealing plastic covers or aluminium seals), a proper plate-roller (e.g. BioRad) or even better a PCR plate sealer (e.g. BioRad PX1 PCR plate sealer) can help prevent cross-well contamination.

E) Store reaction components at the appropiate temperatures outline by the manufacturer. When thawing the NEB WarmStart LAMP kit, we advise thawing at room temperature for 10 minutes with vortex mixing for 5 seconds every 5 minutes. This ensures that any Magnesium sulfate precipiate goes into solution, which is essential for proper HNB colorimetric LAMP detection and proper performance. Check Mastermix after thawing to ensure no precipiate is visible.

F) Colorimetric and fluorescent detection can be performed in the same reaction by simple addition of both dyes (the colorimetric HNB dye and the fluorescent LAMP dye that is provided with the kit).

G) When working with crude samples lysed in QuickExtract, keep samples on ice whenever possible. While we have observed complete inactivation of nucleases after 5 minutes at 95°C, we found considerable RNase activity in biological sample types such as saliva and gargle. Due to the FBS content in Universal Transport Media (UTM), nasopharyngeal swabs in this medium may also contain RNases.

H) RT-LAMP uses 6 primers, two inner loop primers (FIP, BIP), loop primers (LF, LB) and outer bumping primers (F3, B3). A reaction contains all six primers if a single amplicon is tested.

I) Primer choice:
The 'safest' amplicon but not the most sensitive one is As1 (ORF1ab) in our hands
- it does not overlap a CDC amplicon used in the Roche Cobas assays

- it has performed best in our tests regarding false-positives (no false positives)

The more sensitive amplicon is the E1 amplicon.
Disadvantage: The E1 amplicon overlaps the RT-qPCR Cobas E gene assay, thus there is a risk of cross-contamination of diagnostic assays with LAMP reactions.

Optional:
A positive control amplicon detecting the human ACTB gene can be used to confirm the presence of human sample material in the sample, and the absence of general reaction-inhibiting substances in the sample.

SARS-CoV-2 primer sequences:
As1_F3 CGGTGGACAAATTGTCAC
As1_B3 CTTCTCTGGATTTAACACACTT
As1_LF TTACAAGCTTAAAGAATGTCTGAACACT
As1_LB TTGAATTTAGGTGAAACATTTGTCACG
As1_FIP TCAGCACACAAAGCCAAAAATTTATCTGTGCAAAGGAAATTAAGGAG
As1_BIP TATTGGTGGAGCTAAACTTAAAGCCCTGTACAATCCCTTTGAGTG

E1-F3 TGAGTACGAACTTATGTACTCAT
E1-B3 TTCAGATTTTTAACACGAGAGT
E1-FIP ACCACGAAAGCAAGAAAAAGAAGTTCGTTTCGGAAGAGACAG
E1-BIP TTGCTAGTTACACTAGCCATCCTTAGGTTTTACAAGACTCACGT
E1-LB GCGCTTCGATTGTGTGCGT
E1-LF CGCTATTAACTATTAACG

positive control primer sequences (human ACTB transcript)
ACTB-F3 AGTACCCCATCGAGCACG
ACTB-B3 AGCCTGGATAGCAACGTACA
ACTB-FIP GAGCCACACGCAGCTCATTGTATCACCAACTGGGACGACA
ACTB-BIP CTGAACCCCAAGGCCAACCGGCTGGGGTGTTGAAGGTC
ACTB-LoopF TGTGGTGCCAGATTTTCTCCA
ACTB-LoopB CGAGAAGATGACCCAGATCATGT

Materials

› Materials

- › QuickExtract DNA solution 50ml (Lucigen QE09050), store at -20°C
- › WarmStart LAMP Kit (DNA & RNA) (NEB E1700L), store at -20°C
- › Antarctic Thermolabile UDG (NEB M0372L), store at -20°C
- › dUTP solution (NEB N0459S), store at -20°C
- › Primer sets E1 (SARS-CoV-2 E-gene), As1 Rabe&Cepko (SARS-CoV-2 Orf1ab-gene), ACTB as positive control for the garlge; sequences are outlined above; store at -20°C
- › Hydroxynaphtholblue 25g (Merck 1045930025) , store at room temperature under light protected conditions
- › Nuclease free water (not DEPC-treated) 100ml, (Thermofisher AM9938)
- › HBSS - Hank's Balanced Salt Solution without Phenol (Thermofisher 14025092)
- › 1.5 ml Eppendorf DNA low-bind tubes or equivalent product free from RNases and DNases
- › DNase/RNase free PCR-strips or 96-well PCR plates with appropriate seal
- › Plate roller (e.g. BioRad MSR0001) or PCR plate sealer (e.g. BioRad PX1 PCR plate sealer 1814000) to ensure proper sealing of the plates

- 2 heat-blocks (e.g. Eppendorf ThermoStat, with heated lid) or 2 PCR machines
- Optional: Twist Synthetic SARS-CoV-2 RNA Controls Isolate MT007544.1 (Twist Bioscience 102019) for positive control testing, store stock (1e5 copies/µl) or working dilutions (1e3 copies/µl) at -80°C. Dilutions can be temperally stored at -20°C. Avoid multiple freeze and thaw cycler.

Procedure

Preperation of reaction components (Master Mix work area)

1. **Preperation of 10x LAMP primer mixes**

   Thaw 100µM Primer stock and make 100µl of 10x primer mixes. Larger volumes are made by scaling up volumes of individual components

10x LAMP primer mixes

	A	B	C	D	E
1	**Reagent**	**amount to add for E1 mix**	**amount to add for As1 mix**	**amount to add for ACTB mix**	**Comments**
2	F3 (100uM stock)	2	2	2	2µM at 10x
3	B3 (100uM stock)	2	2	2	2µM at 10x
4	LF (100uM stock)	4	4	4	4µM at 10x
5	LB (100uM stock)	4	4	4	4µM at 10x
6	FIP (100uM stock)	16	16	16	16µM at 10x
7	BIP (100uM stock)	16	16	16	16µM at 10x
8	Nuclease free water	56	56	56	
9	Total (µl)	100	100	100	

2. **HNB Dye solution**

   Make 100 ml of 20 mM HNB stock solution from HNB powder (Mw 620.48 g/mol). Be careful when weighing in the HNB powder as it strongly stains fabric.

HNB

	A	B
1	**Reagent**	**amount to add**
2	HNB powder (g)	1.241
3	Nuclease free water (ml)	98.76
4	Total (ml)	100

Sample inactivation and lysis using Lucigen QuickExtract DNA solution

3. Thaw an aliquot of QuickExtract DNA solution on ice and vortex before use. (50µl per patient)

4. On ice, mix 50µl of original sample material (nasopharyngal swab, gargle or saliva) with 50µl of QuickExtract solution in PCR strips or in a 96-well plate; seal the plate (or close the PCR strips with the lids)

   CRITICAL Potentia

5. Heat-inactivate QuickExtract:Sample mix at 95°C for 5 minutes, then cool on ice or store at -80°C

   PAUSE Sample can be stored at -80°C at this point for up to four weeks, potentially longer

HNB-LAMP

6. Prepare the HNB-LAMP reaction mix

   Set-up RT-LAMP reactions in PCR strips or a 96-well plate on ice by mixing the following components in order (top to bottom), then quickly vortex and spin down in microfuge
   CRITICAL This reaction set-up below uses 2µl of sample per 10µl or 20µl reaction. This can be increased to up 20% of the final volume without significant reaction inhibition. That means a maximum of 2µl per 10µl reaction or 4µl per 20µl.
   CRITICAL If multiple reactions are performed, we strongly recommend to prepare a Mastermix for all reaction including a 10% excess. For example, when intending to run 10 reactions, prepare a Mastermix for 11 total reactions. This minimises pipetting errors and ensures enough reagent mixture to be distributed over individual PCR tubes or PCR wells.
   CRITICAL: If performing only colorimetric HNB or fluorescent detection, HNB or fluorescent dye can be replaced with the same volume Nuclease free water. Both can be added in the same reaction tube to record simulatenously real-time fluorescent kinetic profiles and visualise the end-point color change (as shown in our pre-print). This is especially useful when setting up the technique and optimising reaction conditions such as primers and sample types.

RT-LAMP MasterMix with dUTP/UDG

	A	B	C	D	E	F
1	**Reagent**	**per 10µl reaction + dUTP/UDG**	**per 20µl reaction + dUTP/UDG**	**Mastermix for 10 10µl RT-LAMP reactions (+ 10%)**	**Mastermix for 10 20µl RT-LAMP reactions (+ 10%)**	**Final concentration**
2	WarmStart LAMP Kit (2x)	5	10	55	110	1x
3	Primer mix (10x)	1	2	11	22	1x
4	Nuclease free water	1.47	4.94	16.17	54.34	
5	HNB Dye solution (20 mM)	0.06	0.12	0.66	1.32	120 µM
6	50x NEB fluorescent Dye	0.2	0.4	2.2	4.4	1x or 1 µM
7	25 mM dUTP	0.07	0.14	0.77	1.54	0.7 mM
8	UDG (Optional)	0.2	0.4	2.2	4.4	0.02 U/µl
9	**Total***	**8**	**18**	88	198	
10	*Total amount before template addition					

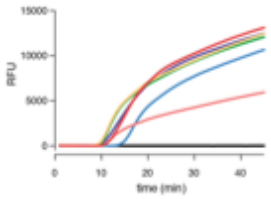
7. On ice, Add 2 µl of heat-inactivated QuickExtract lysate to RT-LAMP reaction and mix carefully by pipetting the reaction up and down.

   CRITICAL This step should be done in a separate work area where no reagents are handled.

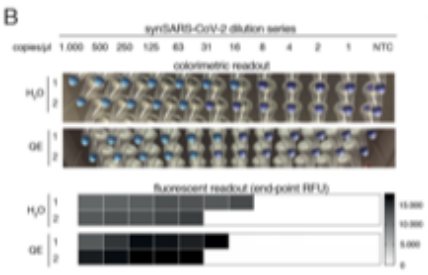
8. If you added UDG, leave the reaction for 5 minutes at room temperature that UDG can cleave any dUTP-containing carry-over DNA contamination.

9. Transfer the reaction to a suitable stable heat-source such qPCR/PCR thermocycler and run reactions at 63°C for 35 minutes.

   For real-time fluorescence data aquisition in qPCR thermocycler, we typically perform 35 cycles at 63°C with 1-minute cycle length and reading at the end of each cycle. NEB fluorescent Dye require data aquisitons using a standard FAM filter (494nm/518nm absorption/emission).



   After 35 minutes run-time, remove reactions and allow to cool briefly at room temperature. Then proceed to inspect reactions visually.
   Negative reactions are purple
   Positive reactions are sky-blue



10. CRITICAL **Discard reactions. DO NOT open and DO NOT run on a gel**